New technologies for high-throughput screening

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To screen efficiently the millions of compounds that are synthesized using combinatorial and automated methods, dramatically improved assay technologies are currently needed. In 96-well microtiter plates, nonradioactive techniques (primarily fluorimetric) and cell-based functional methods have moved to the cutting edge, while clever assays that extract information from large bead-based combinatorial libraries have begun to show considerable promise. In the future, miniaturized assays that break out of the 96-well format will be enabled by innovative technologies for high-throughput screening.

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Abbreviations

CL	chemiluminescence
DTTA-PITC	N1-(p-isothiocyanatobenzyl)-diethylenetriamine-N^1,N^2,N^3,N^3-tetraacetic acid
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
HTS	high-throughput screening
HTS-NT	high-throughput screening new technologies
Ln-TRF	time-resolved fluorescence of lanthanide ions

Introduction

High-throughput screening (HTS) is a well-established method for identifying useful, novel chemical structures. Because of new synthesis technologies such as combinatorial chemistry and automated synthesis, the numbers of new molecules available for screening have exploded in the past few years. Furthermore, a growing number of new targets have begun to emerge from genomics efforts. New technology development in HTS is propelled by the need to evaluate more compounds active against more targets.

Currently, HTS involves a relatively straightforward extension of laboratory-scale assays. Depending on the assay, the adaptation of an assay to HTS can involve either miniaturization (typically, to a 100 µl microtiter plate assay) or automation (generally, attempting to mimic as closely as possible the actions of a researcher in a laboratory assay), or both. Accordingly, the sampling rate that distinguishes 'high-throughput' from slower screening depends on the difficulty of the assay. A typical enzyme-based screen can generally achieve a throughput of 3000 to 5000 samples per day for HTS, while cell-based or other nonbiochemical screens may achieve only a few hundred samples per day to be classified as 'high-throughput'.

This review will cover HTS technologies that are currently in use, as well as those that are in the process of being implemented. Improvements in HTS fall into several categories: simplification of assay development; reduction in per-assay costs; and improvement in sample throughput. Consequently, we shall avoid the prefixes 'ultra-' and 'very' which specifically refer to improvement in sample throughput and use the broader suffix '-NT' to denote the new technologies, in order to define the field as HTS-NT. We have divided the field into three areas of interest: HTS-NT for liquid-phase assays that broaden the applicability of 96-well microtiter plates; HTS-NT for solid-phase binding assays that exploit the solid-phase synthesis characteristic of some combinatorial libraries to improve throughput; and miniaturization technologies for HTS-NT that enable liquid-phase assays in volumes of one microliter or less.

The 96-well microtiter plate technology

Nonradioactive methods

Many laboratories have begun to favor assays that avoid the use of radioactive isotopes. This aversion is due not only to the cost of reagents and to the cost per assay, but also to the inherent limitations on miniaturization of radioactive assays. An assay with, say, a 1000 cpm signal in 100 µl would necessarily have only a 10 cpm signal in a 1 µl assay, requiring 10 000 times as long to count to the same level of accuracy. The principal alternatives to radioactivity are fluorescence and chemiluminescence.

Over the years, several fluorescence methods have been developed to address a wide range of biological assays. In general, using simple fluorescence does not provide adequate performance for HTS, even though it is an inherently sensitive technique. In principle, a single fluorescent molecule can produce thousands of photons such that, in favorable cases, limits of detection have been extended to the single-molecule level. Its primary drawback, however, is susceptibility to background effects, both from the biological milieu and from photophysical effects such as light scattering.

Table 1 compares nonradioactive detection methodologies that have been applied to HTS. One extremely versatile and sensitive method that serves broadly as a replacement for radioactivity is based on the time-resolved fluorescence (TRF) measurements of the rare earth lanthanide ions (Ln-TRF) such as europium (Eu). The first application of Ln-TRF to screening involved antibodies labelled with lanthanides for use in sensitive immunoassays (for

recent references see [1,2]). Because the Eu^{+3} label is at least as sensitive as ^{125}I, which is commonly used in radioactivity assays, this technique has found increasingly broad application as a replacement for radioactivity in HTS assays. Furthermore, many types of assays that have been developed using radioactive labels can be switched to lanthanide-based assays, simply by using different labelling reagents. For example, chemical labelling of free amines has traditionally been carried out using [^{125}I]Bolton–Hunter reagent [N-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate] for which the amine-reactive europium chelate Eu^{+3}-DTTA-PITC serves as a straightforward replacement. In addition, a solution that dissociates europium from the complex in order to enhance the lanthanide fluorescence acts as a replacement for scintillation fluid. Examples of radioactive assays that have been successfully converted to lanthanide assays include those for several types of receptors (both direct ligand labelling [3•] and Eu^{+3}-labelled streptavidin-based detection of biotinylated targets [4,5] and tyrosine kinase assays (using Eu^{+3}-labelled antiphosphotyrosine antibodies [6]). The lanthanide-based system is, however, restricted to assays of pH >7, which ensures the integrity of the chelate.

Further development of LnTRF has been directed toward 'all-in-one' reagents that incorporate the properties of the enhancement solution into the the Eu^{+3} complex, in order to shorten the total time of the assay and to provide a more stable, nondissociable cryptate complex. Achieving comparable sensitivity with these cryptates has proven to be difficult, however, due to the interaction of a single europium ion with multiple excitation 'antennae' when dissociated [7]. Recently, a system has been developed that increases sensitivity by using a high-intensity laser to excite the cryptate [8]. Assays based on FRET (fluorescence resonance energy transfer) from a caged Eu^{+3} to allophycocyanin (APC) further expand the range of the LnTRF method. In this manifestation, it has found many applications in screening, provided a suitable site can be identified for incorporation of the second (APC) label. Incorporation of APC can either be achieved directly (e.g., through a reactive labelling reagent) or using labelled antibodies directed toward a non-obtrusive site in the target [9••,10].

FRET is increasing being used in nonradioactive screening methods [11,12]. A major consideration in choosing an assay based on energy transfer is the distance change that is induced upon ligand binding or enzyme turnover. For energy transfer to be possible, the distance must be less than about 40–50 Å [13]. To put this distance in perspective, 40 Å is approximately the diameter of a protein molecule with molecular weight 26 000 Da. In certain instances (e.g., small peptides that can act as protease substrates), this distance is achievable with mathematical certainty. FRET is of particular utility in assays of proteases, since doubly labelled peptide substrates are generally synthetically accessible and can be obtained from a number of contract suppliers. In other cases (e.g. the binding of a protein ligand to its receptor) the efficacy of the labelling geometry is far less certain.

The use of fluorescence polarization (FP) in HTS applications is growing as a result of the availability of reagents and the development of a 96-well plate reader system [14•,15•,16•]. The technique is well founded in the diagnostics area, serving as the basis for many marketed diagnostics. In this technique, binding events are detected as a loss in rotational mobility of a fluorescence group. The advantages of FP are that only one fluorescent label is needed, and a homogeneous assay can be performed in the presence of background fluorescence. Since the polarization shift taking place upon excitation of the fluor

Table 1

Comparison of methodologies for nonradioactive detection

Method	Advantages	Disadvantages
Straight fluorescence	Simple labelling Simple detection	Sensitivity limited by biological background
Lathanide time-resolved fluorescence (LnTRF)	Sensitive Simple labelling	pH > 7 needed to maintain chelate Requires enhancement solution
Fluorescence resonance energy transfer (FRET)	Large Stokes' shift Homogeneous	Two labelling steps needed Sensitive to distances on the molecular scale
Fluorescence polarization (FP)	Simple labelling Homogeneous	Sensitive to distances on the molecular scale
Homogeneous time-resolved fluorescence (HTRF)	Sensitive Homogeneous Large Stokes' shift	Two labelling steps needed Sensitive to distances on the molecular scale
Chemiluminescence (CL)	Very sensitive Few interferences	Specialized labelling chemistry needed Not versatile

is proportional to the fraction of the total fluor in the bound state order, assays may need to be performed in receptor excess, a situation that is not always practical. Enzymes (primarily proteases, but also kinases and phosphatases) can also be assayed using FP. Compared with FRET, FP is more useful when the targets are large, since larger changes in rotational mobility can generally be observed.

New and potentially interesting approaches for time-domain fluorescence measurements (or two-photon induced fluorescence) are being developed to improve sensitivity under a variety of experimental circumstances [17••,18]. These homogeneous time-resolved fluorescence (HTRF) approaches may be applicable to HTS-NT, but generally require specialized reagents for optimal application as well as new equipment that has yet to be developed. Additionally, Eigen and Rigler [19,20] have suggested that new methodologies employing advances in single-molecule detection can be applied to HTS-NT. It is not yet clear whether limitations in the speed of data collection (at low fluor concentrations) or the saturation of the detection stream (at high fluor concentrations) will limit the range of applicability of HTRF too severely.

Advancements in fluorescent reagents have also been helpful in HTS-NT. Labels that are red-shifted can be distinguished from the biological background (excitation >520 nm) more easily, and are consequently helpful where increased sensitivity is important. The cyanine-based dyes first synthesized by the Waggoner group at the University of Pittsburgh [21,22], are particularly useful in this regard. Reagents that fluoresce in the infrared are also being developed, but have not found significant application in HTS to date, primarily due to lack of available detectors, and insufficient experience with IR labels on the part of biological researchers [23].

Chemiluminescence (CL) is another photometric technique that is applicable to HTS. Detection of CL is a convenient adjunct to fluorescence, since most plate readers capable of measuring fluorescence will measure luminescence as well. This technique has been used predominantly with luciferase reporter genes in cell-based assays and in high-sensitivity enzyme-linked immunosorbent assays (ELISA) employing chemiluminogenic substrates for alkaline phosphatase and horseradish peroxidase [24,25]. Recently, electrochemically generated chemiluminescence has been applied as a sensitive and versatile means of detection, using special, redox-active labels [26•,27,28]. To date, however, this technique has been applied primarily in immunoassay-based detection.

Cell-based functional methods

Functional methods for screening receptor-mediated phenomena have many advantages over traditional receptor binding assays. Essentially, functional screens enable the researcher to discriminate between different binding modes (agonist versus antagonist), as well as to broaden the target base to multiple components of a signalling cascade, regardless of the degree of biochemical characterization of the pathway. Cytofunctional assays, in particular, are finding increasing application to HTS-NT.

One promising methodology is a high-throughput screen based on calcium mobilization. This well-established cytometric assay was developed in 1989 in the Tsien laboratory at the University of California at San Diego [29], but has not been used effectively for HTS because the calcium response is short-lived (on the order of seconds). The development of a high-speed, high-sensitivity imaging system [30], now commercially available, has helped to overcome the limitations of calcium detection and made it a practical HTS methodology. This imaging system is able to collect parallel image data at speeds up to 10 frames per second, at sensitivities that allow detection of calcium transients in cell populations. Concurrent liquid handling and imaging was another key development for the success of this technique.

Another approach toward measurement of cellular assays, particularly assays of membrane potential and intracellular calcium release, is based on the use of a high-intensity laser source with narrow depth-of-field optics which helps to eliminate background. This instrument, known as FLIPR (for fluorescence imaging plate reader), permits rapid parallel stimulation and imaging with high sensitivity [31••]. Like the method described above, this combination of fluidics and imaging allows for high throughput despite the transience of the response.

The yeast *Saccharomyces cerevisiae* has also been useful in developing new HTS methods. It is relatively easy to transfect yeast with human DNA encoding receptors or other components of signal transduction pathways and this has been used to develop an HTS screening system [32]. The signalling pathways in yeast and humans are sufficiently related to permit functional evaluation of human receptors in yeast cells. This method has found particular use in the analysis of human G-protein-coupled seven-pass transmembrane receptors which work well in yeast pathways and provide convenient readouts such as growth (inhibition or promotion) or transcription of reporter genes. Because of the straightforward manipulation of genetic information in yeast, it is relatively simple to generate a family of strains that differ by a single human gene, to facilitate the analysis of screening data. These HTS assays have identified molecules that interact with the human receptors, as well as peptide ligands for orphan seven transmembrane receptors (receptors for which ligands are as yet unknown) [33•].

A technique that involves ligand-dependent transformation of mammalian cells has also been developed. In this method, stimulation of any one of a number of receptors that are transiently expressed in NIH 3T3 (fibroblast) cells confers a growth phenotype [34,35].

Compounds that interact with the stimulated receptor can be identified colorimetrically, because the reporter gene encoding β-galactosidase is amplified during cell proliferation. This method is particularly useful in the functional classification of compound libraries containing both agonists and antagonists.

Methods for screening combinatorial libraries

The advent of solid-phase synthesis as the preferred methodology for generating large combinatorial libraries has presented an opportunity to exploit the immobilization of small molecules to facilitate assay development. Among the early examples of the effectiveness of large libraries as discovery tools was the demonstration that antibody epitopes can be defined by screening combinatorial libraries of peptides. This methodology has led rather naturally to recent developments that involve solid-phase synthetic libraries and their interactions with protein domains [36,37••].

Since solid-phase binding assays do not necessarily reflect the properties of small molecules in solution, additional methodologies have been developed that involve release of the small molecules at precisely defined times and locations. One example of this type of assay is the so-called 'field format' enzyme assay (Fig. 1), in which beads containing a solid-phase combinatorial library attached with a photocleavable linker are immobilized in agarose containing the enzyme of interest.

Additional exploitation of the solid phase has involved selection of compounds through their ability to bind to an immobilized target. Depending on the types of compounds and binding conditions used, this technique can permit the selection of the highest affinity member of a library (since, under excess ligand conditions, this member will displace weaker binding molecules). Alternatively, fractionation and/or direct characterization of the bound molecules can lead to a whole family of binding molecules to allow for the generation of a structure-activity relationship (SAR). This technique has found most utility in identifying peptides that bind specifically to other proteins, for example, MHC class II molecules ([38] and references therein) and phosphotyrosine-binding domains [39].

Figure 1

Field format assay of carbonic anhydrase (M Traverari, T Nichols, CD Carroll, J Burbaum, D Chelsky, unpublished data). The assay was set up in two steps. First, a layer containing bovine carbonic anhydrase (0.1 μM) and low-melt agarose (SeaPlaque™, 0.8%) in sodium phosphate buffer (50 mM, pH 7.4) was layered over two types of beads containing covalently attached carbonic anhydrase inhibitors of the aryl sulfonamide class. On the left side as shown, beads containing an inhibitor with a K_i in solution of 4 nM were used, while on the right side, beads containing an inhibitor with a K_i in solution of 4 nM were used. After the first layer solidified, a second layer containing the fluorogenic substrate fluorescein diacetate was added. A series of photographs taken after the second addition, showing the development of zones of inhibition as a function of inhibitor potency is shown. The numbers at the lower left of each field indicate time in minutes after photolysis of the inhibitors.

Fluorescence-activated bead sorting holds great promise in identifying individual beads that contain compounds of interest. This methodology has been limited to date by technical issues. Commercial cell sorters need to be re-engineered to accommodate bead sorting. The full potential of this strategy may not be realized before the development of specialized instruments designed around the flow characteristics of combinatorial library beads.

Miniaturization

Needs

The technological advances of HTS-NT have resulted in assays that are quicker to set up and execute and are beginning to provide the means to release the full potential of combinatorial chemistry and functional genomics for drug discovery. Miniaturization is vital for full actualization because, in simple terms, the total number of assays required is the product of the number of targets and the number of compounds available for testing. For example, suppose that a full screening effort for a major pharmaceutical company in the year 2000 would require the survey of a deck of 10^6 compounds against 200 targets per year. This supposition approximates a significant increase in both the compound deck and the target pool. In this scenario, 2×10^8 assays of 100 µL, each containing 10 µM test compound with nominal molecular weight of 500 g/mol, would require roughly two million microtiter plates, 20 000 liters of each target solution, and 100 grams of each compound per year. Clearly, the expenses required to extract all the information needed will be exorbitant without a technological overhaul of the screening process.

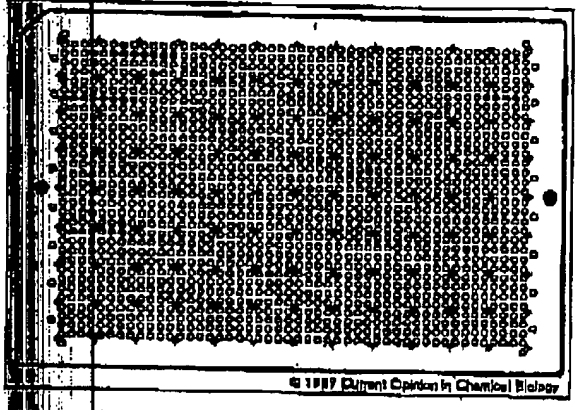
Due to the diverse biochemistry of potential targets, which range from high turnover enzymes to low copy number receptors, not all assays will be amenable to miniaturization. Nonetheless, miniaturization (to the extent that assays remain competent and straightforward to establish) will be embraced in the future. There are presently two broad efforts in miniaturization, classified by the type of container that is needed. In the simpler case, the sample container is an open vessel similar to a microtiter plate well, only smaller. Beyond a certain point, however, miniaturization in an open vessel is impractical because of rapid evaporation of the sample. Thus, miniaturization below the microliter scale requires a closed vessel, which raises issues of loading samples and biological reagents into a tiny chamber.

Open vessel

For conventional well shapes (round- and flat-bottomed), the lower limit for an open container in a laboratory environment is on the order of one microliter (J Burbaum, R Affholter, unpublished data). In this format, more rapid analytical sampling is achieved by packing the microliter-volume wells more densely, in other words, by having more wells per unit area of the plate surface. To maintain compatibility with the 96-well world, the plate density should follow the geometric series $N = n^2 \cdot 96$

where N is the number of wells on the plate and n is an integer, which describes the possible packing densities in a rectilinear array. The balance point of these considerations, microliter volume and suitable packing density, suggests a plate with 1536 wells (n = 4), 1–2 µl in volume. A commercial plate having these properties is the 1536-well HTS plate shown in Figure 2.

Figure 2



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1536-well plate. Each well in a standard 8 row × 12 column 96-well plate has been replaced by a grid of 16 shallow wells of approximately 2 µl total volume. In addition, eight control wells on 9 mm spacing have been situated at the ends of the rows, and four control wells have been placed at the top and bottom of the first and last columns. Through-holes (⊗) that permit alignment of the plate to the plate holder have also been included.

New techniques for detection of fluorescence in the 1536-well format are needed. Conventional plate readers for fluorescence typically read one well at a time, but each 1536-well plate would take about sixteen times longer to read. Because of increased throughput requirements, serial sampling will be unacceptably slow unless substantially accelerated. A more promising approach involves the use of fluorescence imaging, using (for example) sensitive camera-based technologies, to collect data from all the wells at once. Fluorescence imaging is a technique that has seen recent specialized applications in HTS (see above). More general fluorescence imaging techniques being developed will require the development of new strategies to allow for the effects of miniaturization. In particular, the development of robust labelling reagents that fluoresce at longer wavelengths will be needed, with enough versatility to allow for many kinds of fluorescence spectroscopy.

The use of microscopy as a detection tool is another technique that offers more sensitivity. Fluorescence microscopy is a well-established analytical method in cell biology and pathology that is also quantifiable. Until recently, however, limitations in data processing speed

have limited its usefulness in HTS. Clearly, assays that require increased sensitivity would be amenable to microscopy techniques, since microscopy has traditionally yielded fluorescence measurements of single cells.

Finally, techniques for using cell-based assays are needed. HTS will benefit from the miniaturization of the calcium flux and ion channel instrumentation described earlier; in order to exploit the full spectrum of targets, however, the development of new reporter-genes, both for new enzymes and for new fluorescent proteins, will be needed. The utility of various reporter genes in HTS-NT was reviewed recently [34], so will not be covered further.

Closed vessel

In order to develop assays with volumes significantly less than a microliter, a dramatic rethinking of conventional assay strategies is required. This conceptual shift is caused by the necessity of enclosing the assay sample completely to prevent evaporation. Volumetric delivery of fluids in nanoliter volumes without cross-contamination is clearly a problem that must be resolved before HTS-NT in enclosed containers is considered practical. Two new companies have accepted this challenge, and have taken promising approaches toward defining HTS in nanoliter volumes: Caliper Technologies (Palo Alto, California), and Orchid Biocomputer (Princeton, New Jersey).

Caliper's technology uses advanced capillary microfluidics driven electrophoretically to enable rapid and reproducible transport of fluids capable of carrying an electrical current. Recent advances in manufacturing technologies have facilitated the development of complex microfluidic circuits (for a recent review see [40•]) that have been described as laboratories on a chip. To be applicable for HTS-NT, however, assays need to be reworked using electrophoretic or chromatographic separations. Much work is needed to develop robust and versatile separations for biological assay samples in these small volumes, but the payoff in miniaturization and assay acceleration would be striking, particularly with assays involving kinases and proteases for which separations-based assays are currently used.

Orchid's technology integrates synthesis and analysis of library compounds in nanoliter volumes. The challenge of aliquoting organic solvents for synthesis on a nanoliter scale has been met using a proprietary electronic pump that can be used with any solvent. Using fabrication techniques pioneered in the semiconductor industry, nanoliter reaction and analysis vessels are being integrated, such that a single four-inch silicon wafer will support 10^5 separate syntheses/bioassays. Because separations are not required for a successful assay, this technology will provide a strong complement to the miniaturized separations afforded by Caliper's 'lab-on-a-chip' approach. This technology, when mature, will allow for the simultaneous capture of data using a CCD charge-coupled device chip of identical dimensions, allowing for the analysis of many kinds of HTS assays in a rapid, highly-parallel fashion.

Conclusions

Over the next few years we should see a dramatic increase in the ability to analyze large sets of compounds against a large number of targets, as the new technologies for HTS described in this review come into routine use and others not anticipated here are invented. The reason behind this intense effort is obvious: the payoff in terms of new pharmaceuticals discovered will be well worth the investment in new technologies, and will provide the next step forward in the biotechnology revolution. The impact of facilitated compound characterization afforded by HTS-NT will also be significant, with new and more precise tools for dissection of cellular processes becoming readily accessible. New fluorescent reagents, more versatile labelling kits, and miniaturization of assays will all play a role in driving the technology. We can not yet predict the breadth of the impact, but can only predict that the changes in compound discovery methodologies will be fundamental.

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